Title:

Bacteriophage-like particles associated with the gene transfer agent of *Methanococcus voltae* PS

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Summary:

The methanogenic archaebacterium *Methanococcus voltae* (strain PS) is known to produce a filterable, DNase resistant agent (called VTA, for *voltae* transfer agent), which carries very small fragments (4,400 base pairs) of bacterial DNA and is able to transduce bacterial genes between derivatives of the strain. Examination by electron microscopy of two preparations of VTA that were concentrated and partially purified by different methods showed virus-like particles with isometric heads, about 40 nm in diameter, and 61 nm long tails. These particles co-sedimented with the minute bacteriophage ΦX174 in a sucrose density gradient.

Text:

Like all known methanogenic bacteria, *Methanococcus* belongs to the archaebacteria (Fox *et al.*, 1977), a grouping that has revealed new exciting possibilities in the study of early biological evolution (Olsen & Woese, 1997). *Methanococcus* lacks a typical bacterial cell wall. Only a thin protein S-layer covers the plasma membrane. Although cultural work with methanogens (which are strictly anaerobic) is laborious and slow, some genetic information has been accumulating. One of us (Bertani, 1999) has described in *M. voltae* strain PS a system of gene transfer which resembles generalized transduction, except that the bacteriophage component (in terms of viral replication) is defective or absent. The filterable agent, called VTA (for *voltae* transfer agent),

responsible for the transfer, is resistant to DNase and contains 4.4 kb fragments of DNA, derived exclusively or almost exclusively from the bacterial chromosome. VTA is unfortunately very unstable and is very sensitive to certain manipulations, e.g. high speed centrifugal pelleting or suspension in CsCl solutions. We report here the results of two independent attempts to demonstrate the VTA particles by electron microscopy, using VTA samples concentrated and partially purified by different methods.

Technical details concerning mutant strains, media, culturing, measurement of VTA activity (as frequency of transfer of histidine independence, the *his*$^+$ marker), and filtration have been published (Bertani, 1999; Bertani & Baresi, 1987). For **preparation I**, 70 ml of a sterile filtrate from cultures of *M. voltae* strain PS-6 were spun 4 hours at 25,000 rpm, in a Beckman SW28 rotor, at 6 °C, over a cushion of highly concentrated sucrose solution. A volume just above the cushion was collected, applied to a Bio-Rad P-10 Biogel column and eluted (with 0.15 M NaCl, 0.015 M Na citrate, pH 7) to remove most of the sucrose. The fractions expected to contain the VTA activity were pooled and concentrated in Centricon 30 (Amicon, Inc.) centrifugal concentrators. The procedure reduced the original filtrate volume about 1,300-fold. The *his*$^+$ VTA activity in the concentrate was only 9×10^5/ml, corresponding to a recovery of about 3%. The concentrate was stored frozen at -70 °C with 10% glycerol until used for electron microscopy four months later. A phenol extract from this preparation was examined by gel electrophoresis and confirmed the enrichment of DNA of VTA size. For **preparation II**, small volumes of filtrates of strain PS-2, highly concentrated by the PEG-bag method (Bertani, 1999), were layered onto sucrose step gradients (0.9 ml each of 45%, 40%, 35%, 30%, and 0.85 ml of 25% w/w sucrose) in buffer (0.3 M NaCl, 20 mM Tris, 1 mM

EGTA, pH 7.6) and centrifuged (Beckman SW50.1 rotor, 40,000 rpm, 2 hours, 6 °C). Fractions were collected dropwise from the bottom of the tubes. An earlier identical run (see Fig. 2) served as the guide in choosing the fractions corresponding to the VTA peak. These fractions were pooled and further concentrated with Centricon 100 (Amicon, Inc.) concentrators. The total *his*$^+$ VTA activity finally recovered (about 2.8×10^6) was about 20% of the original input, and about 250-fold more concentrated. The VTA activity was well localized on the gradient and well separated from the bulk of UV absorbing material present in the preparation. This preparation was not frozen.

With preparation I, samples for electron microscopy were applied directly to the surface of carbon coated 400 mesh copper grids and allowed to adhere for 2 minutes. The droplet was then removed, the grid surface rinsed with several drops of distilled water, and a negative stain of 0.5% w/v uranyl acetate applied. Excess stain was removed with a capillary and the remainder wicked dry with filter paper. A preparation of bacteriophage T4 was added to the concentrated filtrate to serve as a size reference. With preparation II, the sample (about 0.08 ml) was diluted in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.5) to fill tubes of the Beckman SW41 rotor, and centrifuged for two hours at 30,000 rpm. The invisible pellet was resuspended in 0.25 ml of 20 mM ammonium acetate, pH 7.4. The samples were prepared for electron microscopy by the single carbon method of Valentine *et al.* (1968), in which a layer of carbon is floated onto the sample from a strip of mica, then contrasted with uranyl acetate and mounted on 400 mesh grids. The samples were examined in a Hitachi H-7000 electron microscope at 75 kV.

Our first electron microscopy observations were made on other less concentrated and less pure VTA preparations and showed mostly "membrane vesicles" and bacterial

flagella. The "membrane vesicles" were between 50 and100 nm in diameter and showed in places a regular structural array on the surface (similar to the S-layer, see Jarrell & Koval, 1989). On the other hand, the highly concentrated preparation I showed numerous regular, near-spherical and polyhedral particles about 40 nm in diameter (Fig. 1). These sometimes showed an attached tail, but more often the tail structure was detached. Most of the particles were penetrated by the stain. Similarly, the better purified preparation II showed numerous typical bacteriophage-like particles consisting of a head of diameter about 40 nm and a (usually attached) tail (Fig. 1). No contracted tails were observed. A series of 32 particles from both preparations were measured using T4 phage tails (whose 4.1 nm spacing is known from both electron microscopy and X-ray diffraction, see Karam, 1995) as reference. The averages were: 40 nm for the head diameter (measured perpendicularly to the head-tail axis), 61 nm for the tail length, and 12 nm for the tail width (measured at half length). In all preparations, the largest structures present were the *M. voltae* flagella which have been thoroughly studied (Kalmokoff *et al.*, 1988; Jarrell & Koval,1989) and would offer an internal size-standard, their diameter having been estimated at 13 nm. Unfortunately, their apparent diameter is strongly affected by local staining conditions, flattening, etc. The size of the head of the bacteriophage-like particles would reasonably fit a condensate of double stranded DNA of 4.4 kb, as expected for VTA (Bertani, 1999). Several other structures were observed in preparations I and II: (a) larger, irregular structures ("membrane vesicles") of highly variable size; (b) "small particles", of 9 to 13 nm diameter; (c) circular structures ("buttons") of 17 nm diameter; and (d) fimbriae, about 4 nm in diameter. With the exception of the vesicles, it seems rather unlikely that any of

these other structures may fulfill the requirements for encapsulating 4.4 kb of double stranded DNA.

Additional inferences on the size of the VTA particles could be drawn from a comparison (Fig. 2) of sedimentation rates for VTA, detected as *his*$^+$ marker transfer, and two well studied DNA-containing bacteriophages, P2 and ΦX174 (see reviews by Bertani & Six, 1988, and by Hayashi *et al.,* 1988). The larger P2 bacteriophage has a 33 kb (more exactly, 28.5 kb for the deletion mutant used in these experiments) double stranded DNA molecule in a 58 nm diameter head, with a 1330 × 65 nm tail, while the tailless ΦX174 is 25-30 nm in diameter, with 5.4 kb of single stranded DNA, closer to the size of the virus-like particles described above. As is evident from Fig. 2, the VTA *his*$^+$ activity sedimented at a rate very close to that of the ΦX174 marker.

As discussed by Bertani (1999), 4.4 kb of DNA seem too small to carry all the viral genes necessary for the formation of a structurally complex particle, the control of replication and maintenance in the bacterium, and DNA size-measuring or other cutting specificity in transduction. Other defective systems with virus-like particles carrying host cell DNA are known both for eubacteria and for eukaryotes. To our knowledge, in only two other cases, the gene transfer agent in *Rhodopseudomonas capsulata* (now *Rhodobacter*) (Yen *et al.*, 1979), among eubacteria, and polyoma-related virus (Michel *et al.,* 1967), among eukaryotes, is the size of the DNA fragments incorporated as small as in VTA. The existence in *M. voltae* of larger particles with a larger nucleic acid molecule, representing the viral component, is not excluded, but, if present, they would have to occur in much lower frequency than is the case for classical transduction systems, like P1 or P22 in the eubacteria, or ψM1 in another methanogen, *Methanobacterium*

thermoautotrophicum (Meile *et al.*, 1990). The only other virus-like particles reported to-date for a *Methanococcus* strain are those of Wood *et al.* (1989): tailless, ovoidal, much larger (52 × 70 nm) than ours, and without any known biological activity. While the particles involved in gene transfer in *Rhodobacter* resemble the ones described here, except for their shorter tails, it does not seem that particles of this shape and size have been found to-date in other archaebacteria (see Zillig *et al.*, 1988).

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Legends to Figures:

Fig. 1. Electron micrographs of partially purified, concentrated filtrates from *Methanococcus voltae* PS (see text) showing vesicles, flagella, and virus-like particles. The much larger, tailed particles are bacteriophage T4, added to preparation I as a size reference. Capsid-like particles found in preparation I are shown in the top panel

(indicated by arrows), and in the central row (a tailed particle is indicated by the arrow). Tailed particles found in preparation II are shown in the bottom row.

Fig. 2. Sedimentation of *his*$^+$ VTA and two *Escherichia coli* bacteriophages in a sucrose step gradient (described in the text). Combined data from two tubes spun simultaneously, one tube being loaded with 0.2 ml VTA from strain PS-2, the other with a mixture of *E.coli* phages ΦX174 and P2 *lg del1 del2*. Fractions, collected dropwise from the bottom of the tubes, were slightly different in average volume for the two tubes (0.186 ml and 0.221 ml). Thin solid line, density calculated from refraction measurements for the tube containing the marker phages. Dashed line, absorbance at 260 nm for the VTA tube. The VTA titer in the gradient fractions is given as *his*$^+$ colonies in the standard VTA assay (Bertani, 1999). The VTA activity recovered was 38% of input. Peak titers (pfu) for P2 *lg del1 del2* and ΦX174 were 2×10^6/ml and 6×10^4/ml, respectively.

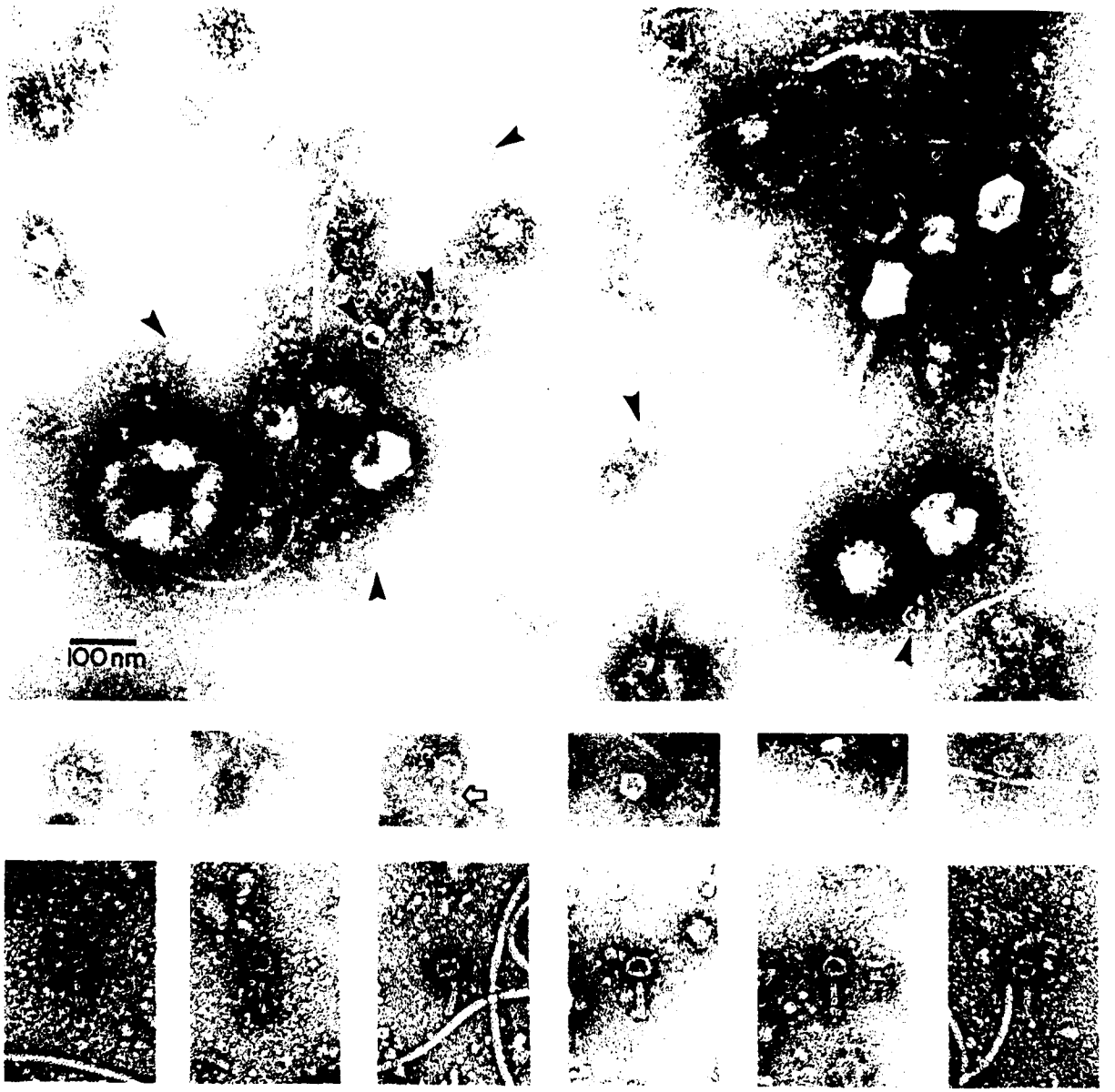

FIG. 1

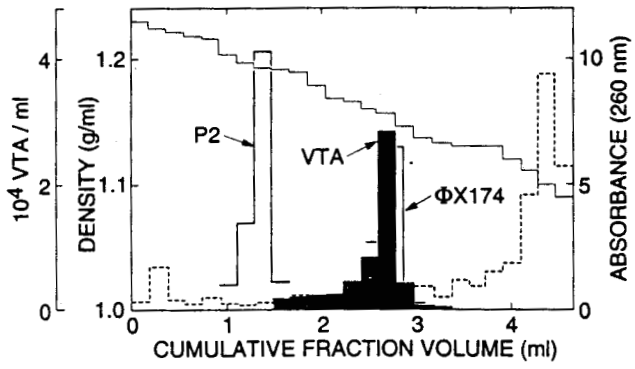
10^4 VTA / ml
DENSITY (g/ml)
ABSORBANCE (260 nm)
P2
VTA
ΦX174
CUMULATIVE FRACTION VOLUME (ml)
0
1
2
3
4
1.0
1.1
1.2
5
10

FIG. 2